Comparison and Standardization of Soil Enzyme Assay for Meaningful Data Interpretation

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Abstract: Data interpretation and comparison in enzyme assays can be challenging because of the complex nature of the environment and variations in methods employed. This letter provides an overview of common enzyme assays, the need for methods standardization, and solutions addressing some of the concerns in microplate fluorimetric assay approaches.

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Professor J.T. Trevors/Editorial Board
Journal of Microbiological Methods

Dear Professor Trevors,

Thank you and the anonymous reviewers for the favorable reviews of our manuscript titled "Comparison and Standardization of Soil Enzyme Assay for Meaningful Data Interpretation". The suggested revisions are made and detailed responses are attached.

Based on your suggestion, we are submitting the revised paper as a letter to the Journal.

Thank you and please do not hesitate to contact us if needed.

With best regards,

Shiping Deng
On behalf of co-authors

***Detailed Response to Reviewers**

Responses to comments/suggestions:

Review comment	Response
1. Revise and resubmit as Letter to the Journal	Accept.
2. e.g.,	Done
3. line 41- this letter	Done
4. line 45 assays	Done
5. MUF write out first time used and place (MUF) in brackets.	Yes, line 42
6. use about not approximately	Done
7. remove actually	Done
8. line 127- reference needed	Done.
9. line 142 remove undoubted	Done

Highlights

- Basic science was sometimes overlooked in application.
- There are concerns on microplate fluorimetric assays for enzyme activities in soil and environmental samples.
- The need and solutions to address methodological concerns are discussed.

Comparison and Standardization of Soil Enzyme Assay for Meaningful Data Interpretation

ABSTRACT

Data interpretation and comparison in enzyme assays can be challenging because of the complex nature of the environment and variations in methods employed. This letter provides an overview of common enzyme assays, the need for methods standardization, and solutions addressing some of the concerns in microplate fluorimetric assay approaches.

Keywords:

Soil enzyme; Bench-scale chromogenic assay; Microplate fluorometric assay; 4-Methylumbelliferone; *p*-Nitrophenol; Method standardization

1. Introduction

There is growing interest in understanding enzymes in the environment, as ecosystems are functionally systems of stored, immobilized enzymes (Burns et al., 2013). Chromogenic and fluorogenic model substrates have long facilitated enzymology research in biological sciences. Chromogenic enzyme assays at the bench scale, e.g., using *p*-nitrophenol (*p*NP) (Tabatabai and Bremner, 1969), have been well tested and widely accepted for use in soils, and are regarded by many scientists as classic methods (Tabatabai, 1994; Nannipieri et al., 2002; Dick, 2011). Microplate format assays were introduced in the early 1990s by several researchers (Wirth and Wolf, 1992; Kremer, 1994; Freeman et al., 1995) and quickly gained popularity, leading to further evaluations on different soils and enzymes (Sinsabaugh et al., 2000; Marx et al., 2001; Pritsch et al., 2004; Kramer et al., 2013). Microplate format assays employ chromogenic or fluorogenic substrates (Wirth and Wolf, 1992; Freeman et al., 1995). The latter gained wider use and acceptance, largely due to high sensitivity and the potential for simultaneous assays of multiple enzymes in the same sample (Marx et al., 2001; Giacometti et al., 2014; Boeddinghaus et al., 2015). Meanwhile, microplate fluorimetric approaches also raised concerns about data accuracy and reproducibility, which stimulated interest in validating data with bench scale assays (Marx et al., 2001; Drouillion and Merckx, 2005; Popova and Deng, 2010). To address some of these concerns, it is important to (1) review basic principles and the fundamental science underpinning enzyme assays; (2) discuss inconsistency and concerns underlying the need for comparison and standardization; and (3) seek solutions. This letter will focus on 4-methylumbelliferone (MUF) microplate assays for enzyme activities in soil and environmental samples, in comparison with *p*NP bench assays.

2. Fundamentals in enzyme assays

Activities of soil enzymes are often determined by incubating buffered soil suspensions with chromogenic or fluorogenic substrate analogues, followed by quantification of the released chromogenic or fluorogenic products calibrated over incubation time. Important properties relevant to quantitative detection of these compounds include their solubility in the reaction matrix and sensitivity to pH for their detection. *p*-Nitrophenol is colorless at pH <5.6 and yellow at pH >7.6. Therefore, quantification of *p*NP in a matrix with pH < 7.6 is not possible, and alkalinization is necessary in this case. MUF is practically insoluble in cold water, but soluble in methanol and ethanol. Therefore, solubilizing MUF model substrates in an organic solvent such as methylcellosolve has been employed (Freeman et al., 1995). However, its sodium salt ($C_{10}H_7O_3Na$, MW 198.2) is freely soluble in water, making the assay more straightforward as most enzyme assays are performed in an aqueous matrix. Fluorescence is also known to be affected by pH and temperature (Lakowicz, 1983), with relative fluorescence signal increasing with increasing temperature, and peaks between pH 10 to 11 (Deng et al., 2013). Consequently, quantitative detection of MUF requires treating standards, blanks, and samples in exactly the same manner and measuring in the same matrix under the same conditions (Deng et al., 2011).

3. The need for method comparison and standardization

Different methodological approaches may be employed in assaying enzymes in soil and environmental samples, such as bench scale vs. microplate format, end-point detection or monitoring changes in product released during incubation, incubation at pH optimal to the enzyme or native to the soil under evaluation, and incubation at physiological temperature of 37^{o}C or environmental temperature of the soil under investigation. Given the variations and

inconsistencies that currently exist, method standardization will undoubtedly lead to enhanced data comparison across studies.

For MUF-microplate assays, common concerns include quenching of MUF in soil and environmental samples (Freeman et al., 1995), the lack of assay replications and standardized procedures for the preparation and pipetting of soil suspension, statistical errors intrinsic to micro-scale assays, pH employed during incubation and detection, and whether or not alkalization is performed prior to fluorescence detection. Freeman et al. (1995) noted dominant fluorescence quenching effects of phenolic compounds in peat. To date, most MUF-based microplate assays do not include assay replicates of soil suspensions. Although replicates up to 16 wells are often carried out, all samples originated from the same soil suspension. The well-recognized heterogeneous nature of soil and environmental samples makes the use of about 0.000833 g soil in each assay well unrepresentative and a source of potential major analytical error, comparing to 1 to 5 g soil per assay in the *p*NP- and other bench assays. Because of the high sensitivity in detection, it is also possible in MUF-microplate assays to quantify enzyme activities by incubating at the pH and temperatures that are native to the environments where samples were taken. Although MUF is known to be pH sensitive with signals peaking between pH 10 to 11, it has been argued that alkalization is not necessary prior to detection because of the high sensitivity (Marx et al., 2001; German et al., 2011). Changes in fluorescence were detected at pH 6.1 (Marx et al., 2001) and even pH 4.5 (German et al., 2011) when fluorescence was very low (Chrost and Kcrambeck, 1986; Deng et al., 2013). However, it needs to be recognized that if incubation temperatures and pHs are not consistent among samples, treatment effects may be obscured and the obtained results are not comparable.

Eliminating alkalization prior to detection makes it possible to quantify enzyme activities by monitoring changes in fluoresce over time. In principle, this is more accurate being based on multiple data points, compared with end-point determination based on one data point. Often, alkalization was accomplished by the addition of NaOH, as in the *p*NP bench assays. Unfortunately, MUF is not stable in the presence of NaOH, requiring detection within minutes (Drouillion and Merckx, 2005) and adding challenges in obtaining reproducible data.

The need for method comparison and standardization is amply evidenced. Surveying articles published since 2000 on β-N-acetyl-glucosaminidase activities in different soils across studies, the reported activities ranged from 8000 nmol kg^{-1} h^{-1} (Grandy et al., 2008) to 1,858,000 nmol kg^{-1} h^{-1} (Creamer et al., 2009) in MUF-microplate assays and from 101 (Ekenler and Tabatabai, 2004) to 1799 nmol kg^{-1} h^{-1} (Acosta-Martinez et al., 2004) in *p*NP-bench assays. The highest reported activities in MUF-microplate assays were over a thousand times higher than the highest reported activity from *p*NP-bench assays. Since these activities originated from different soils assayed by different researchers, it cannot be ruled out that activities of this enzyme vary widely among soil types. However, a comparison of phosphomonoesterase activity using *p*NP- and MUF-based substrate analogues in 15 different soils found that activities in MUF-microplate assays were up to 100 times higher than with *p*NP-bench assays in the same soil (Drouillion and Merckx, 2005). In principle, sensitivity of detection methods should not affect the activities determined. For meaningful data comparison and interpretation, further evaluations of MUF-microplate format assays and method standardization are essential in advancing enzymology research.

4. Solution/recommendation

When assay conditions were carefully controlled, activities of different enzymes in diverse soils measured by MUF-microplate and *p*NP-bench assays were within the same order of magnitude and significantly correlated (Deng et al., 2013; Dick et al. 2013).

1) *Sensitivity:* In the presence of soil suspension, as little as 50 pmol of MUF (compared to 16.28 nmol of *p*NP) can be detected in each assay (Deng et al., 2013). However, the limit of quantification (LOQ) for quantifying enzyme activities using the MUF-microplate approach was about twice of that for the *p*NP-bench approach, suggesting that *p*NP-bench assays are more sensitive when assaying soils with relatively low enzyme activities.
2) *Preparation and pipetting of soil suspension:* The size and shape of the beaker, soil:water ratio, size of the stir bar, and stirring speed and time should be standardized (Deng et al., 2013; Dick et al., 2013).
3) *Quenching*: Developing a MUF standard curve in the presence of the assayed soil and under the identical conditions for assaying samples. For example, fluctuation of room temperature could affect the detection of fluorescence because fluorescence decreases with increasing temperature due to an increase in molecular collisions and subsequent transfer of energy (Guilbault, 1990). As discussed before, "The key for quantitative measures using the MUF-based method is to develop a calibration curve for each soil and each batch of assays using the same reagents under identical assay conditions" (Deng et al., 2011; Deng et al., 2013).
4) *Alkalinization*: Modified universal buffer (MUB, pH 10-12) can be used in the place of NaOH because the relative fluorescence signal of MUF were stable in MUB for at least up to three hours (Deng et al., 2013).

5) *Methods comparison and standardization*: In 2010, the International Organization for Standardization published method ISO/TS 22939:2010 in the effort to standardize fluorometric microplate enzyme assay methods for soil. Unfortunately, some of the raised concerns discussed above are not addressed. The method implied that pH for incubation can be in situ or buffered to optimize activity of the assayed enzyme. However, the effect of pH on the detection of fluorescence was not considered. There was also no mention of replicating the assay utilizing multiple soil suspensions. More recently, Richard Dick led a cross-laboratory comparison of fluorescence microplate enzyme assay, involving all co-authors of this paper (unpublished). Results from this study will further the effort in method standardization.

In conclusion, progress has been made in developing an accurate and standardized approach to enzyme activity measurement, but more work is needed. For example, although changes in fluorescence may be detectable without alkalization, there remains uncertainty as to whether the enzyme activities determined with and without alkalization are comparable. Linking enzyme activities to ecosystem functions demands valid and comparable data, coupled with effective statistical analysis and compelling mathematical models. Method standardization is the first step towards meaningful data comparison and interpretation.

5. Acknowledgements

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6. References

Acosta-Martinez, V., Zobeck, T.M., Allen, V., 2004. Soil microbial, chemical and physical properties in continuous cotton and integrated crop-livestock systems. Soil Sci. Soc. Am. J. 68, 1875-1884.

Boeddinghaus R., Nunan N., Berner D., Marhan S., Kandeler E., 2015. Do general spatial relationships for microbial biomass and soil enzyme activities exist in temperate grassland soils? Soil Biol. Biochem. 88, 430-440.

Burns, R.G., DeForest, J.L., Marxsen, J., Sinsabaugh, R.L., Stromberger, M.E. Wallenstein, M.D., Weintraub, M.N., Zoppini, A., 2013. Soil enzymes in a changing environment: Current knowledge and future directions. Soil Biol. Biochem. 58, 216–234.

Chrost, R.J., Krambeck, H.J., 1986. Fluorescence correction for measurements of enzyme activity in natural waters using methylumbelliferyl-substrates. Arch. Hydrobiol. 106, 79–90.

Creamer, R., Bellamy, P., Black, H., Cameron, C., Campbell, C., Chamberlain, P., Harris, J., Parekh, N., Pawlett, M., Poskitt, J., Stone, D., Ritz, K., 2009. An inter-laboratory comparison of multi-enzyme and multiple substrate-induced respiration assays to assess method consistency in soil monitoring. Biol. Fertil. Soils 45, 623-633.

Deng, S.P., Kang, H., Freeman, C., 2011. Microplate fluorimetric assay of soil enzymes. In: Dick, R.P. (Ed.), Methods of Soil Enzymology. SSSA Book Series 9. Soil Science Society of America, Inc., Madison, WI, USA, pp. 311–318.

Deng, S.P., Popova, I.E., Dick, L., Dick, R.P., 2013. Bench scale and microplate format assay of soil enzyme activities using spectroscopic and fluorometric approaches. Appl. Soil Ecol. 64, 84– 90.

Dick, R.P., 2011. Methods of Soil Enzymology. SSSA Book Series 9. Soil Science Society of America, Inc., Madison, WI, USA.

Dick, L.K., Jia, G., Deng, S.P., Dick, R.P., 2013. Evaluation of microplate and bench-scale β-glucosidase assays for reproducibility, comparability, kinetics, and homogenization methods in two soils. Biol. Fertil. Soils. 49, 1227–1236.

Drouillon, M., Merckx, R. 2005. Performance of para-nitrophenyl phosphate and 4-methylumbelliferyl phosphate as substrate analogues for phosphomonoesterase in soils with different organic matter content. Soil Biol. Biochem. 37, 1527-1534.

Ekenler, M., Tabatabai, M.A., 2004. β-Glucosaminidase activity as an index of nitrogen mineralization in soils. Comm. Soil Sci. Plant Anal. 35, 1081-1094.

Freeman, C., Liska, G., Ostle, N.J., Jones, S.E., Lock, M.A., 1995. The use of fluorogenic substrates for measuring enzyme-activity in peatlands. Plant Soil 175, 147-152.

German, D.P., Weibtraub, M.N., Grandy, A.S., Lauber, C.L., Rinkes, Z.L., Allison, S.D., 2011. Optimization of hydrolytic and oxidative enzyme methods for ecosystem studies. Soil Biol. Biochem. 43, 1387-1397.

Giacometti C., Cavani L., Baldoni G., Ciavatta C., Marzadori C., Kandeler E., 2014. Microplate-scale fluorometric soil enzyme assays as a tool to assess soil quality in a long-term agricultural field experiment. Appl. Soil Ecol. 75, 80-85.

Grandy, A., Sinsabaugh, R., Neff, J., Stursova, M., Zak, D., 2008. Nitrogen deposition effects on soil organic matter chemistry are linked to variation in enzymes, ecosystems and size fractions. Biogeochem. 91, 37-49.

Guilbault, G.G., 1990. Practical Fluorescence, Second Edition, Marcel Dekker, Inc., New York., p. 28.

ISO, 2010. ISO/TS 22939:2010 Soil quality — Measurement of enzyme activity patterns in soil samples using fluorogenic substrates in micro-well plates.

Kremer, R.J., 1994. Determination of soil phosphatase activity using a microplate method. Comm. Soil Sci. Plant Anal. 25, 319-325.

Kramer S., Marhan S., Haslwimmer H., Kandeler E., 2013. Temporal variation of surface and subsoil abundance and function of the soil microbial community in an arable soil. Soil Biol. Biochem. 61, 76-85.

Lakowicz, J.R., 1983. Principles of Fluorescence Spectroscopy, Plenum Press, New York.

Marx, M.C., Wood, M., Jarvis, S.C., 2001. A microplate fluorimetric assay for the study of enzyme diversity in soils. Soil Biol. Biochem. 33, 1633-1640.

Nannipieri P., Kandeler E., Ruggiero P., 2002. Enzyme activities and microbiological and biochemical processes in soil. In: R. Burns and R.P. Dick (eds) Enzymes in the Environment: Activity, Ecology, and Application. Marcel Dekker, New York, USA , pp. 1-34.

Popova, I., Deng, S.P., 2010. A high-throughput microplate assay for simultaneous colorimetric quantification of multiple enzyme activities in soil. Appl. Soil Ecol. 45, 315-318.

Pritsch, K., Raidl, S., Marksteiner, E., Blaschke, H., Agerer, R., Schloter, M., Hartmann, A., 2004. A rapid and highly sensitive method for measuring enzyme activities in single mycorrhizal tips using 4-methylumbelliferone-labelled fluorogenic substrates in a microplate system. J. Microbiol. Methods 58, 233-241.

Sinsabaugh, R.L., Reynolds, H., Long, T.M., 2000. Rapid assay for amidohydrolase (urease) activity in environmental samples. Soil Biology and Biochemistry 32, 2095-2097.

Tabatabai, M.A., 1994. Soil enzymes. In: Weaver, R.W., Angle, S., Bottomley, P., Bezdicek, D., Smith, S., Tabatabai, A., Wollum, A. (Eds.) Methods of Soil Analysis. Part 2. Microbiological and Biochemical Properties. Soil Science Society of America, Madison, WI, USA pp. 775-833.

Tabatabai, M.A., Bremner, J.M., 1969. Use of ρ-nitrophenyl phosphate for assay of soil phosphatase activity. Soil Biol. Biochem. 1, 301-307.

Wirth, S.J., Wolf, G.A., 1992. Micro-plate colourimetric assay for endo-acting cellulase, xylanase, chitinase, 1,3-[beta]-glucanase and amylase extracted from forest soil horizons. Soil Biol. Biochem. 24, 511-519.